The Cytoplasmic Inclusions of the Germ-Cells.

PART V. THE GAMETOGENESIS AND EARLY DEVELOPMENT OF LIMNÆA STAGNALIS (L.), WITH SPECIAL REFERENCE TO THE GOLGI APPARATUS AND THE MITOCHONDRIA.

By

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With Plates 27 and 28 and 6 Text-figs.

Table of Contents.

Introductory.

In the previous part (**20**) of this series of papers I gave a general account of the Golgi apparatus, and showed that the latter was present in the germ-cells and in at least all the more important somatic cells of the Metazoa. Reasons have been advanced which are considered adequate to demonstrate that the molluscan chondrioplasts (Nebenkern rod) are in reality the representative in the germ-cell of the nerve-cell Golgi apparatus (**61**). The molluscan Golgi apparatus has in spermatogenesis been the subject of several exhaustive researches (**19**), and in this section of my work I have described my attempts to trace out this apparatus in the oögenesis, during segmentation, and in the early germ-layer stages. In the same way the mitochondria are followed out.

The oögenesis and early development stages of the organism comprise periods about which no other section of embryology has raised so much discussion and theory, and it is a matter for satisfaction that modern methods should enable us to carry out new researches with a delicacy and certainty hitherto thought impossible. Reference to the table in another paper

(61) will suffice to show that the cytologist has been able to treat his subject from an analytical point of view, which enables him to trace out many bodies with practical certainty.

Previous Work.

No other work has been carried out on the gametogenesis and early development of Limnæa stagnalis, from the point of view of the cytoplasmic inclusions.[1] Several authors have described stages in the development of this Mollusc, or the closely allied Planorbis (48). The oögenesis stages described herein are quite new.

In development one finds the eight-cell stage to consist of four micromeres and four macromeres.

Conklin (7) describes the egg of Limnæa just after maturation as consisting of a clear "well" of protoplasm in the animal pole derived from the nucleoplasm of the burst germinal vesicle, of a finely yellow granular substance close around this clear "well," and beneath a mass of protoplasm with yolk. The clear "well" of karyoplasm afterwards spreads over the animal hemisphere; immediately beneath this clear cap lies the yellow substance, and the nucleus (or pronuclei) lies between the clear and the yellow substances. Conklin shows that the disposition of both the clear and yellow substances undergoes great changes during the time between the first maturation and first cleavage.

Centrifuging just after deposition and before maturation, the egg was found to show three layers—grey, clear and yellow—the clear protoplasm forming the middle layer. The yellow

[1] Since this was written a new paper by Jan Hirschler (58) has come into my hands. This observer has studied the fate of the Golgi apparatus also in Limnæa stagnalis, and has come to the conclusion independently arrived at by me, i. e. that the Golgi elements in the segmentation of the egg are equally distributed and pass through development without losing their identity. The oögenesis stages here given, and the work on the mitochondria are quite new, but I am glad to find that another worker has arrived at the same conclusions as myself with regard to the Golgi apparatus.

zone was the heavier pole of the egg. It contained "yolk"-spherules.

Conklin finds that the injurious effects of centrifuging increase rapidly from the time of the first maturation to that of the first cleavage. Eggs centrifuged during the maturation division usually develop normally; those centrifuged in the resting stage before the first cleavage rarely do. Conklin thinks that this increase in the injurious effects of centrifuging as the egg approaches the first cleavage stage is due to (i) increasing differentiation of the egg, and (ii) decreasing opportunity for readjustment of displaced substances. In all cases where the three substances, clear, grey and yellow, have been sharply separate, the clear protoplasm afterwards diffuses slowly into the grey and yellow zones. Differentiation of the oöplasm takes place mainly between maturation and cleavage. Finally Conklin says that his experiments show that the differently coloured substances of these eggs (Limnæa, Physa and Planorbis) are not "organ-forming" in the sense that each can give rise to only one organ or set of organs. In normal development the clear and grey substances are largely contained in the micromeres, or ectomeres, the yellow substance in the mesomeres or entomeres. But in the centrifuged egg the stratification of these substances may take place at any axis, and yet the form of development may be perfectly normal in every case. In cases the grey material may be cast out of the egg without interfering with normal development. Conklin makes the interesting statement that he got the impression that the grey and yellow substances are mere inclusions in the protoplasm, and that neither is essential to development. The clear substance, which increases rapidly, seems the real protoplasm of the egg, in which the heavier and lighter inclusions are contained. Conklin considers that the yellow substance decreases in quantity during development, being converted into clear and grey (protoplasmic) substances.

Morgan, in the same journal ('Journ. Exp. Zool.,' vol. ix), has studied the effect of the centrifuge on the eggs of

Cumingia (sea-urchin), Cerebratulus (Nemertine), Hydatina (Rotifer), and a fish. His results are hard to interpret from the cell-inclusion point of view, as are Conklin's. With regard to Cumingia he has some interesting remarks to make. He says that "the visible substances of the egg that can be centrifuged are not organ-forming." Morgan considers that abnormal development after centrifuging is not caused by the segregation of the visible substances of the egg. Such abnormal developments are due to mechanical difficulty of transport of nuclei into the mass of shifted yolk, or because of mechanical difficulties of such a mass in the gastrulating cells. Finally, despite such difficulties, Morgan considers that normal development may follow even when the visible centrifuged substances are unequally distributed, and are carried over into the blastomeres, redistribution being thereby prevented.

Technique and Material.

Egg-masses of Limnæa stagnalis were collected from water-weeds or from the sides of small aquaria. Eggs or embryos were either extracted singly from the jelly and fixed in a capsule, or large masses were treated as follows:

(1) They were left in Flemming-without-acetic for from three days to a week, or in Champy's fluid in the same way. They were then washed in running water for one night. After this the entire masses were thrown into the following mixture and left for two days: Chromic acid 1 per cent., 100 c.c.; bichromate of potash 2 per cent., 100 c.c.; nitric acid, 6 c.c. The masses were occasionally shaken up in this fluid (about 25 c.c. should be used), and this treatment dissolved away all the outer capsule and the inner gelatinous substance of the inner egg-capsule, leaving only the membrane, which did not interfere with the sectioning. In this way large quantities were easily done. The mass of eggs and membranes so procured was passed through up-graded alcohols from 50 per cent. alcohol, sectioned in wax, and generally stained in Heidenhain's iron-hæmatoxylin, with or without subsequent treatment in acid fuchsin or orange G.

(2) Masses of eggs were dried on blotting-paper to get rid of as much water as possible, and then fixed in 2 per cent. OsO_4 for a fortnight, according to Kopsch's method. After this they were washed overnight in running water, the outer membranes were separated with needles, and the inner capsules were passed through alcohols, embedded in celloidin and wax and cut into 6 μ sections, and either left unstained or stained in Altmann's acid fuchsin and picric acid.

(3) Egg-masses were first fixed in Carnoy and embedded in celloidin and wax.

After Kopsch's method (No. 2) the subsequent treatment in the chrome-solvent mixture could not be used, because the chromic salts interfere with the specific reduction action of the OsO_4, and the Golgi apparatus was difficult to see. The presence even of a trace of chrome salt tends to spoil the Kopsch reaction.

Warm Flemming-without-acetic or OsO_4 of 2 per cent. penetrated the masses in about a quarter of an hour, and even cold fluid penetrated quickly enough to provide maturation and fertilisation stages if the egg-mass had been just laid before treatment.

The Oögenesis of Limnæa stagnalis.

In Pl. 27, fig. 1, is a germinal epithelial cell of L. stagnalis; it is a flattened cell containing a nucleus with one karyosome and irregular lumps of chromatin; in the cytoplasm is found (Kopsch method) a Golgi apparatus formed of a few separate batonettes or chondrioplasts. No mitochondria could be demonstrated, but I am unwilling to claim that no mitochondria are present. Between the stages in Pl. 27, figs. 1 and 2, the nucleus breaks into filaments and the prophases of the heterotypic division are undergone. These have been given in part in my paper on another mollusc (**18**). By the synizesis stage of the prophases there is found around the Golgi-cum-archoplasmic apparatus a cloud, formed of matter which constitutes the mitochondria; the method of appearance

of this cloud is as already described for Helix aspersa (18). In Pl. 27, fig. 2, the mitochondria are clearly defined, while the Golgi rods are more numerous and the whole apparatus more conspicuous (*G.A.O.*). The nucleus has its chromatin arranged in the manner characteristic of the young oöcyte. Between the stages in Pl. 27, figs. 2 and 3, the archoplasm gradually grows larger, the number of batonettes also increases, and the former becomes constricted, first into larger parts (Pl. 27, fig. 3, *G.A.O.* 1 and *G.A.O.* 3), but soon into smaller parts. I think that it is the archoplasm which is responsible for this primary constriction. The fate of the centrosomes at this period I have been unable to ascertain; possibly, if it does not for the moment altogether degenerate, it detaches itself from the archoplasm and keeps near the nuclear membrane, but I think a centrosome appears later at the time of the formation of the polar bodies. I am inclined to believe that the centrosome becomes detached and lies near the nuclear membrane. Comparing the Golgi apparatus of Pl. 27, fig. 3, with that of Pl. 27, fig. 1, it will be seen that there has been a great increase, the small part marked *G.A.O.* 3 in Pl. 27, fig. 3, being as large as the whole apparatus (*G.A.E.*) in Pl. 27, fig. 1.

The individual batonettes or dictyosomes of the Golgi apparatus do not increase very perceptably in size; it is their number which becomes so large as to cause the apparatus to assume such importance. By the stage drawn in Pl. 27, fig. 4, the Golgi apparatus has spread out through the cytoplasm of one side of the oöcyte (*G.A.O.*), and in the larger oöcyte, in Pl. 27, fig. 5, it has spread on every side of the nucleus. Each Golgi group consists of several batonettes or collections of batonettes which are in process of dividing and then growing. In Pl. 27, fig. 14, the Golgi rod and its archoplasm is shown in process of growth and division.

Eventually in the full-grown oöcyte each Golgi element consists of a sphere of archoplasm, upon one side of which lies the batonette or dictyosome; the latter may be single or multiple; generally in Limnæa it consists of two rods, whose

ends touch at one point. Every part of the oöcyte cytoplasm is strewn with these Golgi elements in a fairly even manner, and every one of these elements has been derived by a process of growth and fission from the Golgi apparatus of the original germinal epithelial cell (Pl. 27, fig. 1, *G.A.E.*).

By the stage drawn in Pl. 27, fig. 2, the mitochondria have appeared as a rapidly-growing cloud of granules embracing the nucleus. If the ovotestis be fixed in Kopsch's method (OsO_4) and stained in Altmann's acid fuchsin and picric acid, the mitochondria stain reddish, while the Golgi apparatus goes quite black; any yolk-granules do not stain, but remain yellowish-brown (with a green tinge) from the osmic acid fixation. In Pl. 27, fig. 5, the yolk would, after Kopsch-Altmann, be greenish-brown to dark brown, mitochondria red, and Golgi apparatus black. There can be no mistake as to these elements, though it is impossible in unstained Kopsch sections to distinguish between egg-yolk and egg mitochondria, both of which are vaguely greenish-yellow to brown. The Golgi apparatus is, however, quite black.

In Pl. 27, figs. 3 and 4, the mitochondria are seen to be in process of growth and dispersal through the cytoplasm. Some of the granules grow faster than the others, and not all the mitochondria remain the same size.

As the oöcyte grows the mitochondria gradually pass evenly through the cytoplasm, and many of the individual grains attain a large size (Pl. 27, figs. 5 and 6). After the stage drawn in Pl. 27, fig. 3, the egg mitochondria grow denser as they become larger, and they no longer remain histochemically of the same nature as the mitochondria in the spermatocytes and spermatids. Nevertheless that these are the egg mitochondria there can be no doubt, as all manner of fixation and staining tests show. The alteration of the older oöcyte mitochondria during the growth period seems traceable to the fact that they become much denser, and therefore are able to respond differently from the more delicate spermatocyte mitochondria. This added denseness is in some way due to the metabolic conditions in the growing

oöcyte—conditions not found in the spermatocyte, which is not surfeited with formed nutritive materials, as is the full-grown oöcyte.

The mitochondria early become impregnated with a yellow pigment, which is destroyed by fixation in chrome-osmium and alcohol, and which gives the fresh egg of Limnæa its bright yellow colour. This pigment (a lipochrome?) is only present in the mitochondria, not in yolk or ground plasma. The full-grown mitochondrium of Limnæa is undoubtedly enlarged by the addition of some lecithin or other fatty matter, apart from its ordinary size. Such materials possibly serve to store energy used in subsequent development, and it is the using up of such material which causes the mitochondria to shrink in size during organogeny.

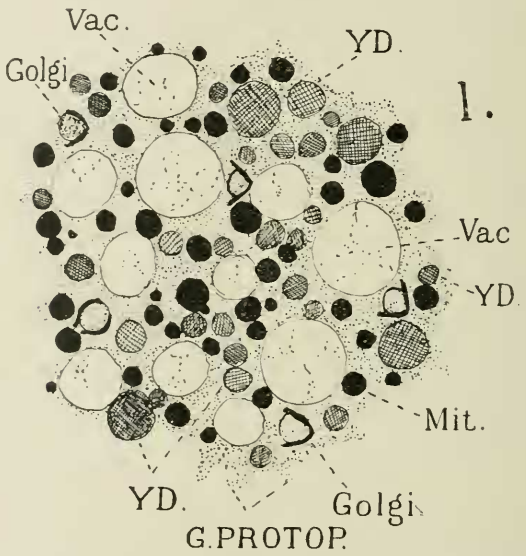
Deutoplasmagenesis.

Deutoplasmagenesis, or the formation of the yolk in the oöcyte, is a simple process. Yolk-discs are often found in the indifferent germinal epithelial cell, but the main formation of yolk-discs takes place after the stage of Pl. 27, fig. 4. While some of the yolk (i.e. the older discs) appears to stain black in iron-hæmatoxylin, fixation in Kopsch or in Flemming-without-acetic acid followed by Altmann's acid fuchsin and picric acid fails to stain the yolk, and leaves the latter greenish-brown, just as it has been coloured by the osmic acid. It is then a very easy matter to distinguish between yolk and the cytoplasmic inclusions. Pl. 27, fig. 5, *Y.C.*, is drawn from an iron-hæmatoxylin-stained preparation. In Pl. 27, fig. 10, is a cell drawn from an unstained Kopsch preparation; Golgi apparatus is black (*G.A.N.*), while yolk is yellowish to brown. In many cases the yolk-discs do not grow much larger than the largest mitochondria, but in other cases they grow about one and a half to three times the size of the largest mitochondrium. Examination of the centrifuged egg in Text-fig. 2 shows this plainly.

Structure of the Cytoplasm of the Ripe Ovarian Oöcyte.

In Text-fig. 1 I have drawn semi-diagrammatically a part of the ripe egg cytoplasm, after the latter is fully differentiated. The cytoplasm of the egg of Limnæa is frothy; this appearance is due to the presence of a large

TEXT-FIG. 1.



Diagrammatic high-power drawing of a part of the cytoplasm to show its structure. The cytoplasmic is vacuolated, and the vacuoles (*VAC.*) contain a coagulum. In the regions between the vacuoles is protoplasm (*G. PROTOP.*) containing yolk-granules (*Y.D.*), mitochondria (*MIT.*), and Golgi elements (*GOLGI*).

number of vacuoles, so numerous as almost to cause the entire egg to have a spongy structure. These vacuoles consist of a fluid substance which almost entirely becomes extracted in finished sections, but which leaves behind a slight coagulum. Whether the vacuoles are oily or watery I find difficult to say, but I think they are watery; the coagulum therefore might not be a fat, but a proteid

substance. These vacuoles appear fairly late in oögenesis, but are always present in the ripe ovarian egg. The "field" of the maturing egg, in which the polar spindles lie, contains no vacuoles, being quite smooth. In the stages following the gastrula vacuoles are hard to find, and are rapidly used up, either as food, or for some process connected with the physiology of development (Pl. 28, fig. 13, *VAC.*, in endoderm). Between the vacuoles of the egg lie the trabeculæ (or sponge-work) of ground cytoplasm, in which are embedded the three elements—yolk-spheres, mitochondria and Golgi grains. This is shown in Text-fig. 1, at *Y.D.*, *MIT.* and *GOLGI;* the ground cytoplasm is stippled (*G. PROTOP.*). In inferior preparations the granules may be carried into the vacuoles, but the best preserved sections show that yolk and other spheres are not normally a part of the vacuole system. This is in opposition to what I found in Helix aspersa (**18**), but I now believe that when yolk or other spheres lie in the vacuoles of eggs sectioned and stained, this is due to mechanical shocks during preparation. Possibly the edge of the knife draws some granules out of position.

Personal Work on Staining Egg and Sperm Mitochondria in Separate Colours.

It has been shown that the egg mitochondria become denser and more chromophil than those of the spermatid. I found it fairly easy, using this fact to guide me, to stain the egg mitochondria black and those of the spermatocyte and spermatid red. The ovotestis was stained in iron-hæmatoxylin as directed in my Helix aspersa paper (**18**), and then differentiated to a stage at which there was still too much hæmatoxylin left in the sections. These were then stained in Altmann's acid fuchsin and picric acid. The egg mitochondria, resisting the differentiation in iron-alum more successfully than the spermatocyte and spermatid ones, were left black before the application of the Altmann; the spermatocyte and spermatid ones were grey to bluish before

the addition of Altmann; the picric acid of the latter completed the washing out of the spermatocyte and spermatid mitochondria, and allowed these to stain in the red fuchsin, while the egg mitochondria managed to hold their hæmatin, and if any fuchsin entered them it only contrived to make them look still darker.

It will be seen that this process, which is capricious, depends on the correct degree of washing out of the ovotestis sections after staining in iron-hæmatoxylin. I found it rather difficult to get the spermatid mitochondria as brightly red as drawn by Held (28) for his Ascaris sperm mitochondria, but this may have been due to a difference in the fuchsin. It should be noted that the ripe sperm tail, like the egg mitochondria, stains black, not red.

The Mitochondria in the Early Development of Limnæa stagnalis.

At maturation, certain stages of which I have been able to examine, the germinal vesicle bursts and the contents of the latter flows upwards to form a zone clear of vacuoles, mitochondria and Golgi rods, wherein the maturation spindles arise.

At this period there is no flow of mitochondria to special regions, all the egg cytoplasm, with the exception of the cap of clear nucleoplasm at the animal pole, being evenly provided with granules, not only mitochondrial, but also yolk. In Pl. 28, fig. 11, is a two-cell stage, the mitochondria being drawn as circles. In Pl. 28, fig. 12, is an obliquely sagittal section of an eight-cell stage; the mitochondria are evenly distributed, even the clear cap of nucleoplasm having spread out flat over the surface of the animal pole.

In subsequent segmentation stages each cell gets a subequal amount of mitochondrial granules. Pl. 28, fig. 13, is a section through the gastrula showing the equal distribution of mitochondria to ectoderm (*ECT.*), endoderm (*END.*) and mesoderm (*MES.*) cells, the mitochondria being drawn as circles.

In subsequent organogeny the mitochondria are still subequally divided. Careful examination of the mitochondria in the unsegmented egg and in the advanced differentiating organ or germ-layer seems to establish the fact that the mitochondria shrink gradually in size pari passu with the differentiation of the tissue. They ultimately reach a minimum size, but do not disappear from the differentiating somatic or germ-cells during any stages I have examined.

The Golgi Apparatus in the Early Development of Limnæa stagnalis.

During maturation the even distribution of the Golgi elements throughout the egg is not altered. In Pl. 28, fig. 11, is a two-celled stage showing the Golgi rods black, archoplasm stippled. In Pl. 28, fig. 12, the Golgi elements, like the mitochondria, are evenly distributed to both micromeres and to macromeres. The rods, just like the mitochondria, lie passively in the cytoplasm, being attracted by neither centrosomes nor nuclei. In all the subsequent segmentation stages this rule is adhered to, and the Golgi elements eventually become distributed to the endoderm, mesoderm and ectoderm cells of the gastrula, as in Pl. 28, fig. 13; here the Golgi elements are black. With the exception of a cell here and there, in almost every case the Golgi rods with their archoplasm lie apparently inert in the cytoplasm of the cells. In the exceptions, such as the ectoderm cell above in Pl. 28, fig. 13, marked *ECT.* and *G.A.*, the Golgi apparatus may lie in a juxta-nuclear excentric position, possibly around a centrosome. In later stages, as the germ-layer cell elements become smaller, the Golgi rods gradually become attracted by either nucleus or centrosome (the latter probably), and become placed in a juxta-nuclear excentric position. In the ectoderm the Golgi rods lie towards the outside of the cell-layer. In division the Golgi rods keep within the zone of the asters. This change in the behaviour of the Golgi elements takes place gradually after the formation of the gastrula.

Centrifuging the Ovarian Egg.

The approach of winter and various duties prevented my complete study of the newly laid egg, but during the November of 1918 I carried out a number of experiments on the ovotestis. The latter in November and subsequent months contains many full-grown oöcytes and provided good material for this study. Ovotestes were removed from the Limnæas, and several were quickly transferred to some salt solution in a centrifuge tube. These were then centrifuged from five to ten minutes at 3500 revolutions a minute on an electric centrifuge.

Immediately afterwards the liquid was poured off and the ovotestes were jerked out into a capsule of fixing solution. Such material was then treated as I had previously done the normal ovotestis. My results were very successful, and have enabled me for the first time, I believe, to study correctly the nature of the layers of the centrifuged egg, already described in the fresh by Conklin. The latter, as has been shown, considered that of the three layers in the centrifuged egg, the bottom yellow and heaviest layer was formed in part at least of "yolk-spheres," then there came the middle "clear substance" and the uppermost "grey substance."

Examined by the best modern methods the egg layers are found to be composed as follows (see Text-fig. 2).

(1) Upper grey substance consists of yolk-discs and a very little protoplasm.

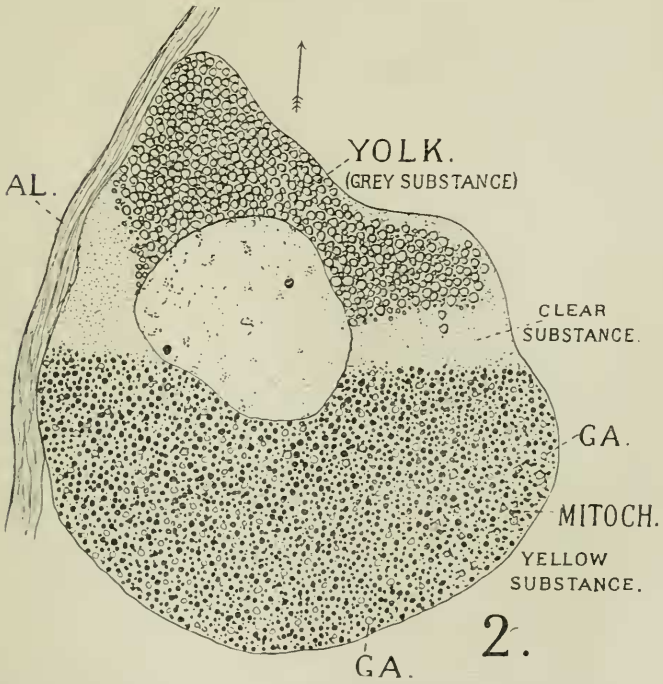
(2) The middle "clear substance" is pure protoplasm without any inclusions.

(3) The lower "yellow substance" consists of the bright yellow mitochondria, and of the Golgi apparatus suspended in protoplasm.

It was noticed that even in the smallest oöcytes containing yolk-discs, the latter came to the top of the cell. With regard to the diffusing of the clear substance into the yellow and grey zones which happens after leaving the centrifuged egg for a little time—a fact noticed by Conklin—I have found

that only those eggs on the outside of the ovotestis (i. e. best exposed to fixative) showed the three clear layers. This was due to the fact that during the time the fixative had taken to penetrate to the inner regions of the ovotestis, the grains dislodged from their normal position by the centrifuge began

TEXT-FIG. 2.



Camera lucida drawing of a centrifuged ovarian oöcyte. *G.A.* Golgi apparatus. *A.L.* Ancel's layer of ovotestis wall.

to regain their normal relations, the yolk sinking, the mitochondria floating up into the clear middle zone.

Conklin found the grey zone (which I consider yolk) could sometimes be completely disrupted from the egg without affecting subsequent development. The fact that this grey substance is yolk explains Conklin's experience. Conklin is wrong I believe in considering the yellow zone contains

"yolk." It consists entirely of yellow mitochondria and of the Golgi apparatus, together with protoplasm.

It was not a difficult matter to fix the centrifuged ovotestis overnight in the modification of Flemming-without-acetic acid, and subsequently stain in iron-hæmatoxylin and van Gieson. The layers were then as follows: Grey (yolk) zone, yellowish-green; clear zone, red; and yellow (mitochondria and Golgi grains) zone, black. With the same fixation and Altmann's stain the zones were as follows: Yolk, greenish; clear zone, yellowish; and mitochondrial zone, red.

Finally I may say that my centrifuge experiments completely uphold my interpretations of the bodies in oögenesis, and such experiments will undoubtedly help observers to clear up doubtful points in studying oögenesis.

Discussion.

Modern research has show that the architecture of the ovum is remarkably complicated, for within its small compass are the potentialities which unfold to form the differentiated embryo and thence the adult. We know that at least two categories of living cell organs, the mitochondria and the Golgi apparatus, are important in oögenesis, and the fate of these structures has been followed out during oögenesis, segmentation and early development; that is during the stages when we know that the potentialities of the ovum are being organised, and are subsequently unfolding themselves.

On the one hand we have a goodly number of excellent observations on the coloured or more or less opaque substances in the fresh egg before, during, and after fertilisation. I refer to such valuable work as that of Conklin and Morgan. On the other hand we have the latest modern work on the cytoplasmic inclusions of the gametes during their formation; there are now a few papers on the mitochondria in early development. It is interesting, therefore, to see how such lines of work compare with each other. I have endeavoured herein to undertake such a comparison, but because of the

fewness of the studies of the inclusions during early development the work has been difficult, and the result somewhat unsatisfactory. The near future will see the elucidation of these questions.

One result of modern work on the elements in the cytoplasm has been to show that the mitochondria and the Golgi rods or granules have the power of binary fission, and in the case of the Golgi rods possibly also the power of multiple fission; for the necessary proof of these facts see **1**, **13**, **18**, **23**, **28**, **29**, **41**, **50**, **52**, **53**, **54**, **56**, and **59** in the bibliography. This result is undoubtedly very important, because it demonstrates that the power of division is not limited to centrosome and chromosomes or nucleus, but is shared by other elements which seem to possess a high degree of morphological independence; both mitochondria and Golgi bodies are able to assimilate, grow and divide in the cytoplasm somewhat as a protist assimilates, grows and divides in its watery medium. I do not believe that either mitochondria or Golgi bodies are symbiotic organisms, as has been claimed for the yellow cells of Radiolaria, but it seems true that the cytoplasmic inclusions have a marked degree of independence. The movements of chondriokonts in the cells of plants and animals are often very elaborate and peculiar, as has been shown by the Lewis's and Guilliermond. Such movements, and even fission, might be directed by special stimuli emitted from the nucleus, but one seems forced to admit that the cell is much more a colony of semi-independent though perfectly regulated elements than was before held to be the case. The exact relationship between nucleus and cytoplasm and between these two parts of the cell and the Golgi elements and mitochondria are at present little known. The fact that both Golgi apparatus and mitochondria have a rôle in gland-cells, such as pancreas and salivary alveoli cells, causes one to favour the view that the cytoplasmic inclusions are not merely growing and dividing at the expense of the ground cytoplasmic and nuclear activity, but are contributing in some way towards the growth and formation of the differentiating cytoplasm; it is to be

remarked, however, that of positive evidence we have very little either way at the present moment.

The complete demonstration of both Golgi apparatus and mitochondria in all animal cells appropriately examined is a fact of cardinal importance to zoologists, and leads us to consider that by the aid of experimental methods a flood of light may in the near future be shed on the obscurities surrounding our knowledge of the modus operandi of the cell organs in various vital phenomena.

Definition of Organ-forming Substances.

In order not to cloud the following remarks it is necessary for me to state exactly what is meant by the words "organ-forming substances." Speaking generally, these words simply mean those materials in the egg-cell which, as in ascidians, become segregated into special regions and ultimately come to form organs or parts of organs—such would be yolk which in many animals comes to lie in the endoderm. But when one considers, it seems likely that in the latter case the yolk is not the only endoderm-forming substance which is situated in the embryonic endoderm; I cannot believe that the cytoplasm of the latter is not different from that of the ectoderm or mesoderm. The substances which confer on the endoderm-cell its quality of "endodermness" are presumably derived from the nucleus at some stage of oögenesis or development, but such substances are in reality the "organ-forming materials" in the truest sense of the word. Whether

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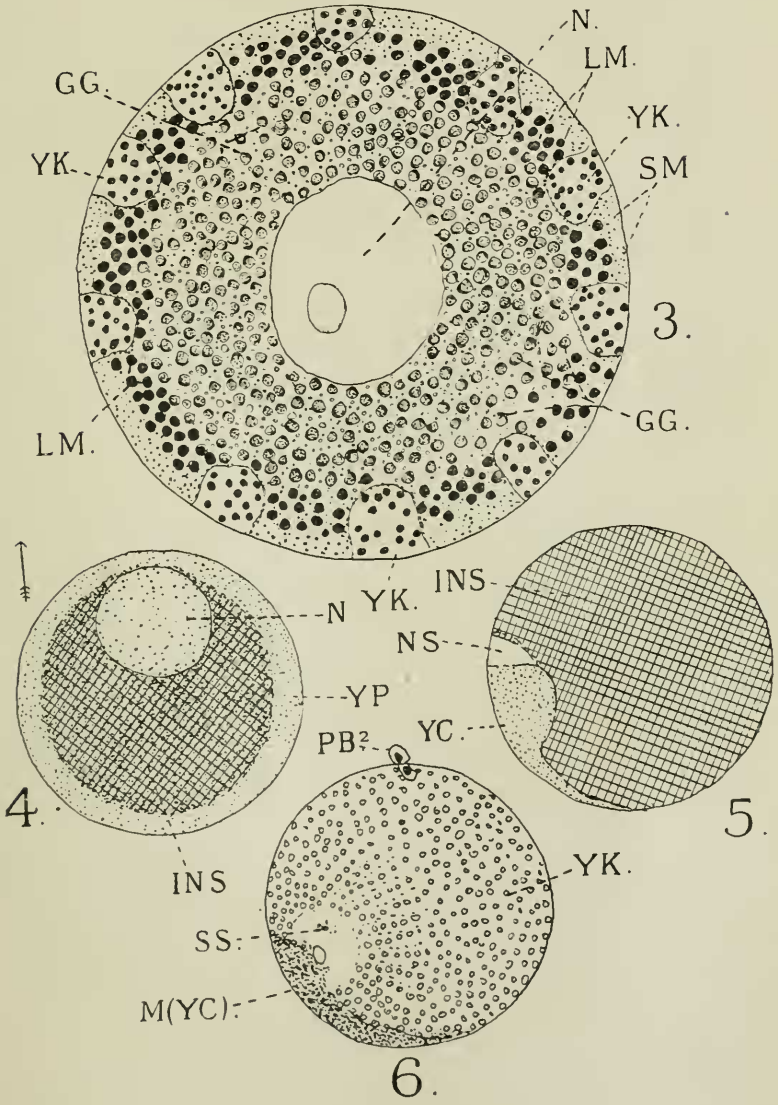
Text-figs. 3–6.

3. Full-grown ovarian oöcyte of Ciona (diagrammatic after Hirschler). *G.G.* Golgi apparatus. *L.M.* Large mitochondria. *S.M.* Small mitochondria. *Y.K.* Yolk pockets. 4. Schematic figure of Cynthia oöcyte, after Conklin. *N.* Nucleus. *Y.P.* Yellow-pigmented cytoplasm. *I.N.S.* Inner region. 5. Maturing egg during fertilisation. *N.S.* Clear substance (karyolymph). *Y.C.* Yellow crescent. 6. Ciona egg at same stage after Duesberg. *YK.* Duesberg's "yolk," which Hirschler calls Golgi elements. *S.S.* Clear space around male pronucleus. *M.* (*Y.C.*). Mitochondria in yellow-crescent region.

TEXT-FIGS. 3–6.



the mitochondria and Golgi elements are carriers of these suggested inner substances, or themselves constitute these substances, is a moot point which is further discussed below; but it is believed that some distinction should be made between such organ-forming substances as yolk and between the more subtle bodies which I have assumed to exist in the ground cytoplasm. Yolk, fat, pigment, and possibly also the mitochondria and Golgi apparatus might be looked upon as purely "nutrient" organ-forming substances, engaged in supplying materials for the work being carried out by the "definitive" organ-forming substances. These definitive or positive organ-forming substances might be what one well-known writer has assumed to be special enzymic bodies. In the following remarks the words "organ-forming substances" are taken to mean both the "nutrient" and "definitive" organ-forming substances unless otherwise stated.

Mitochondria, Organ-forming Substances, and Idioplasm of the Cytoplasm.

MacBride (**34**), in his recent valuable work "Text-book of "Embryology—Invertebrata," describes organ-forming (definitive?) substances as materials emitted from the nucleus during the ripening of the egg—materials which confer on the cytoplasm a definite character. Organ-forming substances are, according to this interesting writer, most plausibly to be regarded as of the nature of hormones or ferments.

By the majority of workers the hypothetical substance called "idioplasm," the physical basis of inheritance, is thought to be identical with the chromatin. We are driven then to ask, What relationship is there between the idioplasm and the organ-forming substances? and between both these and the cytoplasmic inclusions, Golgi apparatus and mitochondria?

Again, MacBride writes (**35**): "We have been gradually led to view the nucleus as a storehouse of all the characters of the species, and to look for the cause of the first differentia-

tions seen in development in the modification of the cytoplasm through the emission of substances from the nucleus." MacBride (**35**) quotes the work of Schaxel (**49**) and of myself (**16**) as support that such emissions can be seen in prepared specimens of developing oöcytes. Schaxel's work has been shown to be wrong in so far as this observer describes emanations of solid chromatin particles from the nucleus, through the nuclear membrane into the cytoplasm (see Beckwith **3a**, and **18**), while my own work, done some years ago by imperfect technical methods, cannot be interpreted as supporting Schaxel. In my work on the frog (**16**) I showed that a basophil cloud could be seen to appear around the nuclear membrane at a certain period. Further work shows that this cloud is formed of mitochondria which were present in the young cell, but which at a certain period of activity of the nucleus of the metamorphosing cell begin to grow and become denser. Nevertheless, it must be pointed out that in reality MacBride's interpretation of the fact is correct, because few would care to deny that the growth and changes of mitochondria at this period were not directly due to emanations from the nucleus. The point to be noticed is that the mitochondria are not chromatin, and do not usually appear in themselves directly to be due to emanations from the nucleus, but their primary stimulation and growth are probably initiated by fluid substances passed out from the nucleus. These substances are not chromatin apparently, nor can they be seen in the form claimed by Schaxel.

In a paper on the snail (**18**) I showed that, with the Flemming-without-acetic acid and iron-hæmatoxylin technique, the nucleus of the indifferent Helix germinal epithelial cell, when becoming "progerminative," could be seen to be capped on one side by a cloud of almost structureless stainable matter (see **18**, Pl. 31, figs. 11 and 19). I regard this for the time being as perfect evidence, in MacBride's sense, of emissions of substances from the nucleus into the cytoplasm, but I cannot say how further improvement in technique will lead us to interpret the peri-nuclear cloud.

The cloud drawn by me in the figures already mentioned is due to the coagulation by the fixative and subsequent staining by the hæmatoxylin of some substance which, intra vitam, must have occupied that region of the cell. Whether this substance existed intra vitam in the form of grains, of bulk colloid, or of a filamentous cloud, as drawn by me, is of no real importance to this part of the discussion. The really vital fact is that such a cloud was present in some form during the life of the cell, and that this cloud is developed in such a relation to the nucleus as to allow of no other interpretation but that it comes under the direct influence of the nucleus. But I agree with Miss Beckwith (**3a**) in this: "There is no evidence of formed material passing through the nuclear membrane into the cytoplasm either early (Schaxel) or late (Smallwood) in the growth period." By the term "formed material" I would mean chromatin or other visible "solid" particles or grains as drawn by Schaxel,[1] and this apparently is Miss Beckwith's view.

Duesberg considers that the mitochondria do not represent the organ-forming substances, but that the mitochondia of the egg represent that idioplasm (**10**) which is situated in the cytoplasm. Unlike Duesberg, at present I do not see my way clear to distinguish between idioplasm and organ-forming substances, but I am prepared to endeavour to distinguish between the *definitive* organ-forming substances and the mitochondria.

With regard to Duesberg's interesting views something may now be written. This observer, in his latest paper (**10**), no longer dogmatically insists that the tail of the sperm is that part of the male cytoplasm which takes part in fertilisation, and he shows every sign of preparing his ground for a

[1] In certain Insecta the nucleus of the oöcyte appears to extrude some of its chromatin in the form of grains, which constitute the 'secondary nuclei." The latter are distinct from either mitochondria or Golgi apparatus; the secondary nuclei are treated in a forthcoming paper of this series. Dendy has described extrusion of chromatinic matter in the oöcyte of a sponge ('Quart. Journ. Micr. Sci.,' vol. 60 1914–15).

complete rejection of his former view. But, while Duesberg has doubts with regard to the idioplasmic function of the middle-piece of the sperm, he now adopts a new view: it is that the mitochondria of the egg represent that part of the idioplasm supposed to be situated in the cytoplasm; while to explain the difficulties with regard to the mitochondrial part of the sperm, he assumes that the gametes are really inequivalent, and that the idioplasmic function of the egg mitochondria is possibly not shared by that part of the spermatozoon.

Duesberg, however, fails to give any explanation as to the function of the sperm-mitochondria. The very constancy of their presence in all sperms shows that they must have some function. Duesberg quotes the views of Jenkinson, Schreiner and Broman on the supposed inequivalence of the gametes, and uses this as support for his view that the egg and sperm mitochondria have a different function.

It seems true that in some forms at least (Molluscs and Nematodes) the egg mitochondria are much denser than the mitochondria of the spermatid or spermatozoon, but this greater density of the egg mitochondria is possibly connected with the differing metabolic conditions in the egg, as contrasted with the sperm, where there is very little storage of food materials. Moreover, one would expect the sperm (not the egg) mitochondria to be denser, since denseness might be associated with concentration.

Duesberg considers that the idioplasm of the cytoplasm of an egg might be located in the mitochondria. More recent researches (Weigl, Hirschler, Gatenby) show that in some cases the bodies in the cytoplasm are not all mitochondria, many being derived from the Golgi apparatus—a distinct cytoplasmic organ, which, however, is somewhat related to the mitochondria in some of their chemical reactions. Duesberg should now explain what place the Golgi apparatus fulfils in his conception of the location of the idioplasm, and he should bring it into apposition with his views on the mitochondria, for both Golgi apparatus and mitochondria are alike definite cytoplasmic organs evidently sharing in the formation of the

ground-protoplasm of the egg. Further, it seems certain that some definite relationship exists between these two cytoplasmic organs; in Limnæa and other Mollusca (Helix, Arion) and in Hydractinia (Beckwith) the Golgi apparatus is small in bulk when compared with the mitochondria, while in Hirschler's Ciona the Golgi apparatus is very large in bulk and the mitochondria relatively smaller. (Miss Beckwith's "pseudo-chromatin granules" are, I believe, the Golgi apparatus, for her description of them corresponds very closely with that of the Golgi apparatus in mollusc eggs.) It therefore seems probable that the smallness in bulk of one sort of cytoplasmic inclusion may be made up by the largeness in extent of the development of the other—a fact which points to some relationship of function between the two.

If one fixes the ovary in Carnoy's fluid (absolute alcohol, acetic acid and chloroform), and subsequently treats in alcohol and xylol, one sweeps almost everything out of the cytoplasm, and leaves the latter smooth; the cell then is seen to consist of a nucleus and a ground-cytoplasm. To my mind the ground-cytoplasm left after this operation is the place of location of the cytoplasmic idioplasm, and definitive organ-forming substances. In a given germ-layer of an embryo I consider one might get in the cytoplasm of the cells several groups of materials. One might have an organ-forming substance distinct from the pure cytoplasm of the species, one would have in addition yolk, and the two categories of cytoplasmic inclusions. The relationship between, and common identity of, the organ-forming substance and the pure cytoplasm is a matter of doubt. When I use the term "pure cytoplasm," I conceive that cytoplasm which must be common to all cells of the body no matter what their function, and which is, after the nucleus, in itself the basis of the largest and smallest characteristics of that organism. In special organs this cytoplasm may be temporarily or permanently altered, or infused with special substances necessary for the organ to fulfil its functions. In this conception the mitochondria, instead of taking a major place as prime substances of

heredity, take the minor position as substances important in some parts of growth and general metabolism. But a great deal more work will need to be done properly to clear the various issues above mentioned. This I readily admit.

The mitochondria and the Golgi grains may also take part in the storage or elaboration of substances which may be drawn upon to provide energy during segmentation and organogeny; but these views are very different from that of Duesberg, who wishes to identity with the mitochondria functions directly connected with the hypothetical idioplasm. Moreover, that the mitochondria have some function either as the storers of energy-producing materials, or the providers of such, is, I consider, made more probable by Duesberg's own demonstration that the mitochondria take a great part in the formation of the myoplasm (muscles) in Ciona. One cannot but believe that the works of Guilliermond on plants (22–25), Dubreuil (12) on mitochondria and fat, and Arnold (2a) on the pancreatic zymogen granules and the mitochondria all argue strongly against the Meves-Duesberg view.

Under this section it may safely be concluded that present researches on the mitochondria in gametogenesis and development lead us strongly to believe that the mitochondria are unconnected with idioplasm or other hypothetical hereditary substances. Their function seems to be to elaborate certain materials which are utilised in the up-building of the gamete.

Mitochondria, Golgi Apparatus, and the Organ-forming Substances in Ascidia.

Jan Hirschler (29) describes the oögenesis of severa ascidians, and shows that the egg contains yolk, mitochondria and Golgi granules. In Text-fig. 3 is a diagrammatic drawing showing the final arrangement of the granules in the ascidian egg (Ciona). At the periphery of the egg lie a number of pockets containing yolk (*YK.*), and in the same region lie large mitochondrial grains (*L.M.*), about four or five deep. More inwardly and around the nucleus one finds a very large

number of Golgi granules (*G.G.*). Interspersed between the latter there are smaller granules which are identical in histochemistry with the larger Golgi grains, and mixed up with the peripheral mitochondrial grains are much smaller mitochondrial elements (*S.M.*), also identical in their histochemical reactions with the large mitochondria. Hirschler's main points are as follows:

(1) The mitochondria lie peripherally and are not mixed up with the Golgi elements.

(2) The Golgi elements form the bulk of grains in the egg, and lie internally to the peripheral mitochondrial elements.

(3) There are smaller Golgi and mitochondrial grains lying in the special regions occupied by their larger fellows.

(4) Yolk is scanty and lies in pockets on the periphery of the egg.

Hirschler gives satisfactory evidence of the difference in morphology and histochemistry between Golgi elements and mitochondria, both of which he has traced out from the earliest germ-cell. Personally I find nothing to doubt in his description of the oögenesis of the ascidian.

Conklin's work on the "organ-forming substances" in Ascidia is too well known to need lengthy description. MacBride has given a careful account of Conklin's work in his recent 'Text-Book on Embryology.' In Text-fig. 4 is a diagrammatic copy of one of Conklin's figures of the mature oöcyte. The periphery of the egg (*Y.P.*) is occupied by a rind of "yellow protoplasm" (compare with Text-fig. 3). The inner part of the egg contains much more opaque materials (*I.N.S.*); at *N.* is the nucleus. During maturation and fertilisation the nucleus bursts, and the karyolymph flows upwards and forms a cap on the animal pole; this material (or part of it) and the yellow rind of cytoplasm now flow downwards and around the sperm-aster, as in Text-fig. 5; *Y.C.* is the yellow cytoplasm (mitochondria) or myoplasm, and *N.S.* the clear cytoplasm (karyolymph). The inner more opaque substances (Golgi granules ?) are at *I.N.S.*

Duesberg finds the yellow-cresent substance (*Y.C.*) to be

mitochondria. In Text-fig. 6 is a diagrammatic copy of his interpretation of the unsegmented maturing egg. The substance at *X.*, corresponding to Hirschler's Golgi elements, Duesberg calls "yolk." Duesberg seems to be in agreement with Hirschler with regard to the grains (yellow pigment) in the outer rind of the egg; both observers find it to be mitochondrial. Since Duesberg does not pay any attention to the Golgi apparatus and has not used the correct technical methods, one can understand his calling Golgi granules "yolk."

Shortly, Hirschler's or Duesberg's work shows:

(1) The outer peripheral layer of the egg (yellow cytoplasm with pigment) contains the mitochondria.

(2) The rest of the egg is mainly occupied by Golgi elements (Duesberg's "yolk").

(3) The yellow crescent (myoplasm) is formed of protoplasm, in which lies the bulk of the mitochondria.

Unfortunately Hirschler does not discuss his work in the light shed by Conklin's studies on the fresh ovum of ascidians. The interpretations of the "plasms" of Conklin's ascidian egg are still much clouded, and the matter is far from being settled by either Hirschler or Duesberg. Both observers contribute valuable evidence. Finally it may be stated that Hirschler and Duesberg have brought forward evidence which shows that certain of the "organ-forming" regions in the fresh ascidian egg owe their differentiation to a definite segregation in those special regions of granules which are probably the mitochondria and the Golgi apparatus.

Golgi Apparatus.

There can be no doubt now that the Golgi apparatus and mitochondria are distinct and separate from each other. The attitude of Duesberg in his comprehensive 1912 review can only be upheld in view of the work of Hirschler, Weigl, Perroncito, Nussbaum, Golgi, Cajal and myself by disregarding the facts. In his paper on Ciona, Duesberg (**10**) uses no Golgi apparatus method such as that of Kopsch or

Cajal, and further adherence to his standpoint is impossible. When Duesberg uses the proper methods I have no doubt that he will find the Golgi apparatus to be distinct from the mitochondria.

The reader who is not specially acquainted with the latest literature on the Golgi apparatus and mitochondria may be assured that while there is distinctly good evidence of a relationship of chemical constitution between Golgi apparatus and mitochondria, there is now quite sufficient evidence which has been independently produced by several reliable workers that the Golgi apparatus is a distinct entity in all active cells of the metazoan; in germ-cells especially is the Golgi apparatus distinct and evidently separate from any mitochondrial apparatus. In the case of Monocystis ascidiæ, Hirschler ('Anat. Anz.,' vol. xlvii) found that there was a typical Golgi apparatus distinct from the mitochondria. I have little doubt that other Protozoa will also be found to possess these two distinct categories of cell-organs.

The study of the Golgi apparatus in "zoological" material has been somewhat neglected. Curiously enough, some zoologists seem to consider that this cell-organ is rightly neglected; such a valuable paper as that of Hans Held (**28**) is incomplete, because he neglected to study the Golgi apparatus, previously shown to be present in both egg and ripe sperm of Ascaris by the excellent Polish observer Jan Hirschler (**59**). Held's remarks on Ascaris may therefore be interpreted with Hirschler's paper. In all probability the few Golgi batonettes present in the Ascaris sperm are, like the mitochondria, carried over during fertilisation, and continue afterwards to grow and divide; whether, however, they spread throughout the egg cytoplasm like the sperm mitochondria it is impossible to say. The Golgi apparatus, this present paper shows, is during oögenesis equally scattered throughout the cytoplasm of the ovum, and in segmentation the blastomeres, before the process of gastrulation, are each provided with a portion of the original apparatus of the germinal epithelial cell. The out-spreading of both Golgi apparatus and mitochondria of Limnæa we now

know to be, among other things, a preparation for the subsequent disposal of parts of these elements to every cell of the morula, and thence of the embryo and adult organism.

In Mollusca of the Pulmonate type, such as Limnæa, Helix or Arion, and in Paludina, the Golgi apparatus seems to be sloughed off the sperm during spermateleosis, and can take no part in fertilisation and thence in heredity. The Golgi apparatus of Molluscs seems to be passed on from generation to generation through the ovum. Subsequent work might reveal a Golgi apparatus in the ripe sperm, but so far my own researches have failed in this respect, and I do not believe that Golgi elements are present in the ripe mollusc spermatozoon. Weigl (**53**) gives a figure showing the presence of the Golgi apparatus in the sperm of Cavia. Weigl's work is illustrated by convincing microphotographs, and may be recommended to those who desire to deny that the Golgi apparatus and mitochondria are not separate entities.

In Lepidoptera (**17**) I described the presence of certain bodies specially in spermatogenesis, which I traced ultimately to vesicles in the spermatid; I described these vesicles as running together to secrete a granule which was the acrosome of the sperm. I have now little doubt that these sickle-shaped rods —called by me acroblasts—are really the Golgi apparatus of Lepidoptera. I have re-examined all my material and have made more preparations, and can come to no conclusion other than that they form the acrosome and do not slough off. Several friends who have examined my sections have come to the same conclusion. The matter is still being examined by me. Weigl (**53**) has described similar bodies in a Sphingid, but does not follow out their fate. Casteel's (**5**) bodies in Argas are likewise possibly Golgi apparatus, and they also behave very like my bodies in Lepidoptera. Further work is needed to clear up these questions with reference to Arthropoda, and especially Insecta.

Our knowledge that the Golgi apparatus (and the mitochondria) spreads out throughout the egg cytoplasm during oögenesis might be interpreted in two ways: it may be con-

sidered that this spreading out is to enable the activities of the Golgi rods to be felt in every corner of the cytoplasm, or that the spreading out of the apparatus is only in preparation for subsequent segmentation of the egg. I think both interpretations are true, and there seems little doubt that the two categories of cytoplasmic inclusions whilst spreading out are taking some part in the building up of the oöcyte.

It was previously pointed out that the spermatid mitochondria are immensely smaller in bulk than the egg mitochondria; exactly the same unequal relationship exists between egg Golgi apparatus and spermatid Golgi apparatus. The explanation of this may be that the cytoplasmic inclusions, being concerned in metabolism, are naturally proportionately small in the small spermatid cell and proportionately large in the oöcyte, which contains so much more nutrient and formed matter. While it can be shown that the chromatin matter in egg and sperm is equal in bulk (e. g. the pronuclei at fertilisation), this relationship has only been shown to apply to the mitochondria at the same period in one case—Ascaris megalocephala (28).

The great importance of the Golgi apparatus may be gauged when one remembers that every sort of metazoan cell carefully examined has been found to possess the typical apparatus. Every mitosis or karyokinesis is also, as well, a dictyokinesis, or a nearly equal sorting out of the Golgi rods between the daughter-cells (20). Moreover, it seems probable that when the possibly universal occurrence of Golgi apparatus in Metazoan, protozoan and plant cells is recognised widely, and the attention of more workers is brought to bear upon these problems, some relationship between the amphiaster and the Golgi rods or granules may be discovered, and the real function of the cytoplasmic inclusions definitely ascertained.

The main fact which it is desirable to emphasise in this section is that a Golgi apparatus has been described in every animal order, and is throughout of the same general type. In my previous article I gave some description of the morphology of the apparatus. It nearly always consists of a sphere of

archoplasm, such as is found around a centriole (centrosome), upon which lie several more or less deeply curved batonettes or little rods. In mitosis the rods are sorted out whole—a process called by Perroncito dittokinesis or dictyokinesis; each daughter-cell gets about half of the original number of rods; in some cases the rods are branched and may fuse to form a reticulum.

Centrolecithality, Telolecithality and Alecithality with regard to Yolk-discs and Mitochondria.

The yolk in Limnæa is not massed on one side, nor does it lie in the middle: it is evenly scattered through the cytoplasm; this has been called a homolecithal egg. In Limnæa there is some evidence that the vacuoles in the protoplasm, already described, are more numerous in the vegetative hemisphere; that they ultimately mainly come to lie in the large endoderm cells seems certain. There is every likelihood that the large mitochondria, which are possibly partly laden with food material of a lipin nature, and which are less easily destroyed by certain techniques than the delicate yolk-spheres, might be mistaken for the latter. It must be noticed by embryologists that such yolk as one finds in molluscs is very delicate, and does not stain black in iron-alum hæmatoxylin. The bodies found in many invertebrate eggs, which stain black in hæmatoxylin, are nearly always mitochondria. In another paper I have carefully entered into the histo-chemistry of yolk and mitochondria (**61**).

What it is necessary to point out here is that in the eggs of molluscs (Helix, Paludina, etc.), insects (Apanteles, Sphinx, etc.), amphibians (Rana, Triton), mammals (Mus, Lepus), the mitochondria extend throughout the egg and are not especially segregated into regions; undoubtedly this applies to the majority of animals, examples of which have been left out in the above list.

In some forms, such as Ciona or Cynthia (**29**), it is equally certain that the mitochondria (and Golgi apparatus) are arranged in a special manner. For these rare examples the

paper of Hirschler on Ascidian oögenesis may be consulted (29). It is quite certain that in the majority of forms studied, the mitochondria and Golgi apparatus do not take part in producing any definite polarity of egg substances such as yolk does. Even in the markedly telolecithal ovum of Rana the mitochondria are spread through the entire cytoplasm, and it would not be possible to say that the upper hemisphere had a smaller quantity than the lower, or vice versâ.

Embryologists not specially acquainted with recent advances in cytology are in the habit of calling any round, stainable granules of the egg cytoplasm yolk. This is wrong, and what such workers call yolk may often be really Golgi granules and mitochondria; the latter are quite different, being formed of a protoplasmic basis impregnated with some phosphatide. Yolk is a dead storage substance and not a true cell organ as is the mitochondrium or Golgi granule or rod. Neglect to distinguish between yolk, mitochondria and Golgi elements only introduces confusion; in another paper this matter is treated more fully from the practical view-point.

The Mitochondria in Metazoan Spermateleosis.

In spermateleosis, or the metamorphosis of the spermatid into the spermatozoon, the mitochondria vary remarkably in their behaviour. After a perusal of the latest work on the subject, together with the results I have myself ascertained, I find that five main classes exist:

(A) In the amœboid spermatozoon of Ascaris and in the Decapod sperm the mitochondria remain morphologically unchanged, though grouped loosely in a special region of the spermatozoon.

(B) In the Mammalian type also the mitochondria generally maintain their individuality, but become grouped around the upper part of the axial filament often to form a spiral, and generally in a specialised region between the head and second centrosome (so-called middle-piece).

(C) In many Insecta the mitochondria fuse to form a more

or less elaborate coil, or macromitosome, losing their individuality.

(D) In Pulmonate Mollusca the mitochondria do not bodily form part of the sperm-tail, but seem to be drawn upon as reserve material for the secretion around the axial filament of a new layer of mitochondrial nature. This is probably what occurs in Peripatus (**44**).

(E) In many other Mollusca the spermatid mitochondria are few in number, and bodily fuse to form a solid structure around the axial filament. This also occurs in some scorpions (Wilson, **54**).

It is noteworthy that while this variation in behaviour of the mitochondria take place, the nuclear phenomena are throughout fairly regular.

From the above descriptions it will be evident that during spermateleosis in some animals the mitochondria are, as far as we know, morphologically as well as chemically unaltered, while in others it is almost equally certain that not only does a morphological change take place, but, what is more important, a chemical one. In Ascaris the sperm mitochondria, being unaltered, and being carried into the egg, may pass on from generation to generation. In the mollusc of the pulmonate type the spermateleosis stages are different; the mitochondria lose their individuality, and do not bodily form a part of the tail. In the case of insects it is equally clear that a complete change comes over the mitochondria at spermateleosis (**17**).

I believe that the above will be found to explain the varying behaviour of the mitochondria during fertilisation in various groups of animals. Further reference to this important question is made in the following pages.

The Behaviour of the Sperm Mitochondria after Introduction into Ovum.

In most animals properly studied it has been established that the entire sperm penetrates into the egg at fertilisation.

The only possible exception seems to be the Nereidiformes, where Lillie (**33**) and Just (**30**) both conclude that the middle-piece, i.e. the mitochondrial part of the sperm, is left outside and never penetrates the egg-membranes. In the majority of animals studied by skilled cytologists the sperm after entry is seen to break up into three pieces (at the least)—the nucleus, the centrosome, and the mitochondrial part behind the head centrosome. The fate of the nucleus and the centrosome need not detain us here. The fate of the mitochondrial matter so introduced by the male element is our immediate concern. In Phallusia (**41**) and in Ascaris (**40**) Meves describes the sperm mitochondria as being attracted into the fertilisation area (in the case of the ascidian, around the centrosome), and taking direct part in fertilisation, in the case of Ascaris at least, by fusing with the egg mitochondria, as the sperm nucleus fuses with the egg nucleus. In Phallusia Meves did not show that the ♂ and ♀ mitochondria fused, but he establishes that the ♂ mitochondria do grow and continue actively dividing in the egg cytoplasm after the disintegration of the sperm. In the case of Echinus, Wilson (**55**) and then Meves showed that the middle-piece does not become active after entry of the sperm. Meves (**42**) carried the matter further in two papers, and demonstrated that the middle-piece of the sea-urchin sperm may eventually become haphazardly segregated entire into a cell of either the animal or vegetative pole; the fate of the middle-piece in the sea-urchin is quite different from that of the ascidian. In the Lamellibranch *Mytilus edulus*, Meves (**43**) showed that the mitochondria enter the egg, but he was unable to demonstrate any subsequent activity as in Phallusia. In Ciona, Duesberg (**10**) failed to show any activity of the ♂ mitochondria; in the mammals no activity of the middle-piece has been shown, and as far as known it may be segregated whole to one or the other blastomere of the two-cell stage. The suggestion that the blastomere of the two-cell stage which gets the middle-piece becomes the formative (embryonic) part, while the other becomes the trophic

part, is interesting, but does not accord with the hypothesis which accounts so well for the origin of identical twins; for, if the middle-piece made a difference in the blastomeres, one would not expect the twins to be identical. Finally, the case of Ascaris may be mentioned: Held (**28**) recently shows that by staining in molybdate hæmatoxylin, and then in Altmann's acid fuchsin, the ♂ mitochondria become red, the ♀ keep black, and the two sorts can be followed out during fertilisation and even to segmentation. The "specificity" in coloration merely depends on the washing out of the first stain from the less dense ♂ mitochondria. Held shows that the ♂ and ♀ mitochondria of Ascaris do not fuse as claimed by Meves, and that after entry of the sperm the ♂ mitochondria grow, divide and multiply very rapidly, till ♀ and ♂ granules are of a like quantity.

Tentative Explanation of the Behaviour of the Mitochondria during Fertilisation.

We have seen that in Ascaris and possibly also in such an ascidian as Phallusia the sperm mitochondria begin to grow and divide after transference to the egg, while in such a form as Parechinus the mitochondrial part of the sperm does not fragment and grow in the same way. Assuming that subsequent researches will prove that in certain groups this growth phenomenon of the mitochondria of the sperm holds good, it may be well to attempt to give some explanation other than that the process is connected with the transmission of cytoplasmic factors. The explanation which occurs to me is as follows: Believing that the mitochondrium is a living plastid-like body of semi-independent automatic functions of purely metabolic nature, and that its main function in the sperm is that of storing (or taking from the surrounding medium) enough energy-producing materials to enable the sperm to live and move till it reaches the egg, I consider that after the sperm enters the egg the mitochondrium merely begins to undergo the vital metabolic phenomena in the new

cytoplasm to which it has been carried. It is as if a bacterium was transferred from an old culture medium to a fresh one.

The reason why the Ascaris sperm mitochondria begin to grow when transferred to the egg is that they are provided with a new field of activity and at once begin to utilise it. It may, then, be asked why the mitochondria do not go on dividing while in the middle-piece, since their vital functions are assumed to be semi-automatic in the sense that they are not directly due to another body. The answer to this would be that the functions of the mitochondria can be controlled and directed by stimuli emitted from the nucleus—a supposition which few will care to doubt—and that the sperm mitochondria are temporarily under the influence of some substance which keeps them from dividing, but not from functioning as energy-providers. If it be objected that such a view would not apply to the sea-urchin fertilisation where the middle-piece remains inert, it may be explained that in all probability the spermateleosis stages leave the Echinus mitochondrial matter in a modified state, which inhibits a recovery of growth after entry of the sperm.

In Ascaris the mitochondria are not altered, but probably in the sea-urchin the ripe middle-piece is not really formed of normal mitochondrial matter, but of modified material which dies on entering the egg, or which is unable to recover its former qualities. In this connection the evidence collected on p. 476 may be consulted, when it will be seen that my contention that the mitochondrial matter in the ripe sperms of different animals is rarely formed in the same way, or exists in the same quality, will be seen, I believe, to have been proven. Hence the varying behaviour of this mitochondrial matter after transference to the egg cytoplasm. Studying pulmonate molluscan spermateleosis stages in Kopsch's method (OsO_4, fourteen days), I found that at the time the sperm-tail was being formed the mitochondria went much darker than at any other stage, showing that a chemical change had overtaken them. (See also **19**, p. 248, for further evidence.)

Possible Objections to the above Tentative Explanation.

I have assumed that the mitochondrium consists of two sorts of substance, one the protoplasmic living basis, the other the lipin or phosphatide. That the mitochondrial granule is not metaplasm, but a living entity, I conclude from these facts: It had long been believed, and in several cases has now been actually demonstrated, that the mitochondrium has the power of dividing by binary fission. As remarkable evidence of this I may mention Held's recent work (**28**), where it is shown that the mitochondria of the male element divide and grow rapidly inside the cytoplasm of the egg (refer also to Wilke, **56**, Wilson, **54**, and Fauré-Fremiet, **13**).

Secondly, the behaviour of the mitochondria in the spermateleosis stages of spermatogenesis can only be explained by the assumption that these bodies have the power of spontaneous movement (**56**, **17**). If it be objected that such evidence does not necessarily allow one to conclude that the mitochondrium is living substance, it can be pointed out that it is exactly similar evidence that enables us to conclude that the chromosomes are living. Further evidence that the mitochondrium consists of these two parts is got by fixation experiments, where it is found that mild fat-solvents remove some part of the mitochondrium and leave a residue.

In addition, it is now firmly believed that the mitochondrium is formed mainly of a phosphatide; this is merely the name of a dead substance extracted from the cell, and it is clear that such a substance must, *intra vitam*, have been accompanied by some other material which would enable the above-mentioned reactions, such as that of binary fission, to take place.

We may now inquire into the position in which these assumptions lead us. In the first place, believing that the mitochondria, unlike fat, yolk, or pigment masses, are able to undergo movements which we can only interpret as vital, and that such movement is due to the fact that the basic substance

of the mitochondrium is living protoplasm, it is clear that in such a case as the fertilisation in Phallusia or Ascaris, sperm protoplasm must be introduced into the egg through the instrumentality of the sperm mitochondria. This being so, some may take the view that the Benda-Meves theory is proven—for Ascaris at least. I believe that such is far from being the case. There are many who take the Darwinian view that "inheritance must be looked upon as merely a form of growth" (8a), but it is to be noticed that the supposed function of the mitochondria as elaborators of certain "fatty" substances which partly enable the cell cytoplasm to grow does not necessitate our believing that these are in themselves the substances of heredity, or that the mitochondria can in any way influence the substances of heredity. The conclusion arrived at above (p. 468) with regard to the mitochondria in development has already been stated: it is that those materials which must be present in the cell and which bear in themselves the hereditary factors when not actually located in the nucleus are found in the ground-cytoplasm of the cell. That such is the case I consider indicated by the evidence already produced with regard to the erratic behaviour of the mitochondria in the germ-cell cycle, and by the centrifuge experiments on eggs.

Finally it may be explained that while I believe that the plant or animal plastid or mitochondrium has as its basis living protoplasm, it is conceived that this protoplasm might be specialised for metabolic or trophic functions, as apposed to reproductory ones. A further advance in these views will only be made possible by further research, and I am prepared to admit that there are many points which are most obscure, and which occasionally may even be found to give more or less specious support to the mitochondria-idioplasm hypothesis. My present views are due to my dissatisfaction with the evidence offered by such observers as Meves and Duesberg, but were the latter to produce further and more convincing facts I would still be prepared to abandon my present attitude. But that such evidence will be produced is doubtful, and at all

events it is quite certain that the function of the mitochondria is of secondary importance in the life of the cell as compared with the nucleus. This conclusion is further supported by observations on the behaviour of the mitochondria during mitosis, gametogenesis and organogeny, treated elsewhere (**61**).

Present General Conclusions with Regard to the Cytoplasm in Fertilisation.

It is now known that the following cell organs possess the power of fission and are self-propagating units : chromosomes, centrosome, mitochondria and Golgi apparatus. I overlook the cases in which a centrosome seems to appear out of the nucleus. Mitochondria and Golgi rods or granules have a very high degree of morphological independence in most if not all parts of the developmental cycle of the organism. The exact relationship between the nucleus and the Golgi elements and mitochondria and degree of independence of the latter are not known, but it seems unlikely that the nucleus would not have a great measure of control over all the cytoplasmic inclusions. There is now strong probability that the ripe sperm in some forms contains, in addition to nucleus, not only mitochondria but Golgi apparatus, and it seems likely that in cases both categories of inclusions are carried over to the egg. The latter has been shown to contain the same elements as the spermatid, only in much larger quantity.

In a few cases the sperm mitochondria, after introduction into the egg, are found to grow and divide, somewhat like bacteria in a nutrient medium. Though it is possible that the Golgi apparatus of the Cavia sperm and of the Ascaris sperm is introduced into the egg, so far no evidence has been brought forward as to their subsequent activity.

Finally, it is probable that the behaviour of the sperm mitochondria in various animals is so variable, that it is unsafe to look upon them as bearers of hereditary factors of any kind. The same will probably apply to the elements of the Golgi apparatus, which are more rarely carried over to the egg.

Summary.

Oögenesis.

(1) In the germinal epithelial cell of Limnæa stagnalis a Golgi apparatus is present. It is excentric and lies around the archoplasm, consisting of a number of rods (chondrioplasts or dictyosomes, dittosomi).

(2) In the progerminative oöcyte mitochondria appear at a very early stage, but it is not known whether they exist in the indifferent germinal epithelial cell. The mitochondria lie at first in the zone of the Golgi apparatus.

(3) The rods of the Golgi apparatus divide by binary fission and keep growing in number. The archoplasm upon which they repose gradually becomes divided into regions; these regions again subdivide till each Golgi rod is discrete and provided with a small part of the archoplasm, which it partly embraces. As each Golgi rod divides transversely the archoplasm does not divide. The latter only divides by binary fission after it has become studded with a number of rodlets.

(4) The Golgi apparatus gradually, from its excentric position, spreads completely throughout the egg cytoplasm, and in the full-grown oöcyte is evenly distributed here and there in all parts of the egg cytoplasm. No segregation into special regions was noticed.

(5) The mitochondria, from their excentric position near the Golgi apparatus, grow, divide, and spread evenly throughout the cytoplasm. The mitochondria are not all the same size; this is apparently due to the fact that some granules grow larger and more quickly than others.

(6) While the egg mitochondria grow much larger than the spermatid mitochondria, it has been shown that the individual Golgi batonette or rodlet never grows beyond a certain size. The difference between the Golgi apparatus of a young oöcyte and a full-grown ovum lies, not in the fact that the Golgi rods of the latter are individually very much larger (if at all) than those of the former, but mainly in the fact that the rods have increased enormously in number by binary

fission. The individual Golgi rodlet of spermatid, young and old oöcyte are approximately subequal in size.

(7) Deutoplasmagenesis, or the formation of yolk, does not begin very early; the first yolk-discs make their appearance after the Golgi elements and mitochondria have progressed far in the process of spreading throughout the growing oöcyte. The yolk-discs do not appear in any special region of the cytoplasm, but eventually become evenly spread out. The discs at first are very small, and later grow some two or three times larger than the largest mitochondria. In Flemming-without-acetic (overnight) + iron-alum hæmatoxylin, yolk goes dark brownish-green, mitochondria black.

(8) Towards the end of oögenesis the cytoplasm gradually becomes filled with vacuoles of a fluid nature. These leave a coagulum on fixation, but most of the vacuole is empty. The granules in the cytoplasm only abnormally lie in these vacuoles; yolk, Golgi elements and mitochondria lie in the trabeculæ between the vacuoles.

Spermatogenesis.

(1) The spermatogenesis of Limnæa stagnalis agrees in the main with that of other Pulmonata which have previously been studied (**18**, **19**).

(2) No micromitochondria were discovered. In spermateleosis there is a mitochondrial residue sloughed off. The mitochondria do not bodily form the tail of the sperm; the tail of the sperm appears as a new formation of mitochondrial matter around the axial filament. The spermatid mitochondria are drawn upon to provide material for this process; as a result they alter chemically.

(3) With Kopsch's method (OsO_4 of 2 per cent., fourteen days) at the time of the formation of the mitochondrial tail, the mitochondria go dark brown, as apposed to the much lighter colour (i.e. power of reduction of OsO_4) of the mitochondria in the spermatocyte and early spermatid. They also become more resistant to injurious fixation.

(4) The Golgi apparatus is sloughed off during spermateleosis.

(5) In the fully formed spermatozoon, the nuclear head of the sperm is very small as compared with the immense length of the mitochondrial tail.

Centrifuge Experiment.

The egg centrifugalised before maturation has three layers: the upper or grey substance is yolk; the middle or clear substance is protoplasm; the lower and largest layer (yellow substance) is protoplasm, in which are suspended yellow mitochondria and Golgi elements.

Segmentation.

(1) In segmentation of the egg the mitochondria are equally divided, and keep so in organogeny stages examined.

(2) The same applies to the Golgi apparatus.

(3) In organogeny neither mitochondria nor Golgi apparatus disappear.

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EXPLANATION OF PLATES 27 and 28.

Illustrating Mr. J. Bronté Gatenby's paper on "The Gametogenesis and Early Development of Limnæa stagnalis."

Lettering.

A.L. Ancel's layer of ovotestis wall. *AR.* Archoplasm. *C.C.* Clear cytoplasm. *ECT.* Ectoderm cell. *END.* Endoderm cell. *G.A.* Golgi apparatus element. *G.A.E.* Golgi apparatus of epithelial cell. *G.A.O.* Golgi apparatus of oöcyte. *G.A.N.* Golgi apparatus of nurse- (yolk-) cell. *G.A.S.* Golgi apparatus of spermatocyte or spermatid. *G.E.* Germinal epithelium. *G.R.* Golgi rod. *J.B.* Juxta-nuclear body. *M.* Mitochondrium. *MA.* Macromere. *MES.* Mesoderm cell. *MI.* Micromere. *N.* Nucleus. *P.B.* Polar bodies. *T.* Sperm-tails (mitochondria). *VAC.* Vacuole. *Y.* Yolk-disclet. *Y.C.* Yolk- or nurse-cell. *X.* Line of division of archoplasm.

PLATE 27

Fig. 1.—Indifferent germinal epithelial cell, showing Golgi apparatus. Kopsch.

Fig. 2.—Later progerminative cell after "appearance" of mitochondria. Kopsch-Altmann.

Fig. 3.—Young oöcyte showing growth of Golgi apparatus. F.w.a.; iron-hæmatoxylin.

Fig. 4.—Little older oöcyte showing outspreading of Golgi apparatus and mitochondria. Ditto.

Fig. 5.—Half-grown oöcyte, Golgi apparatus and mitochondria; yolk drawn as circles. Ditto.

Fig. 6.—Nearly full-grown oöcyte, showing juxta-nuclear body (*J.B.*) clear cytoplasm (*C.C.*), Golgi elements (*G.A.O.*), and mitochondria (*M.*). Yolk drawn as circles throughout the cytoplasm. Ditto.

Figs. 7 and 8.—Spermatocyte and spermatid respectively showing Golgi apparatus and mitochondria. Ditto.

Fig. 9.—Ripe sperm, drawn to same scale as oöcyte in Fig. 6. Smear.

Fig. 10.—Nurse-cell, showing Golgi apparatus, yolk, and heads of spermatozoa adhering to cell. Kopsch unstained.

PLATE 28.

Fig. 11.—Two-cell stage, showing equal distribution of mitochondria and Golgi apparatus elements. Kopsch.

Fig. 12.—Obliquely sagittal section through micromeres and macromeres of eight-cell stage showing equal distribution of same elements. Kopsch-Altmann.

Fig. 13.—Median section through gastrula, to show equal distribution of all cell elements except oily (?) vacuoles (of endoderm). Mitochondria drawn as circles, Golgi apparatus elements black. Cells of all three germ-layers shown. Kopsch-Altmann. (6 com. × $\frac{1}{15}$th semi-ap., reduced $\frac{1}{3}$.)

Fig. 14.—Semi-diagrammatic scheme of method of division and multiplication of Golgi elements.